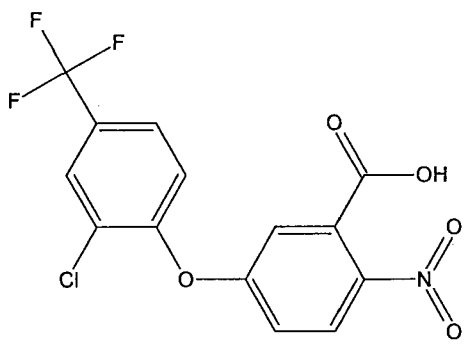
1.0 INTRODUCTION

1.1 Scope and Chemical Structures

BASF analytical method A9206 was developed for the analysis of Acifluorfen and its metabolites (Acifluorfen-Amine, Acifluorfen-Acetamide, and Des-Carboxy-Acifluorfen) in water. The limit of quantification (LOQ) of the method has been established at 1.0 ppb for acifluorfen and its 3 metabolites in water. [Note: The method was extended to a lower LOQ for the aciflurofen during an Independent Method Validation (ILV) conducted by JRF America. The extended LOQ for aciflurofen was established as 0.1 ppb (µg/L).] This method satisfies EU guidelines SANCO/3029/99 rev. 4, SANCO/825/00 rev. 7 and US EPA guideline OCSPP 850.6100.

The chemical structures for the reference materials are summarized as follows:

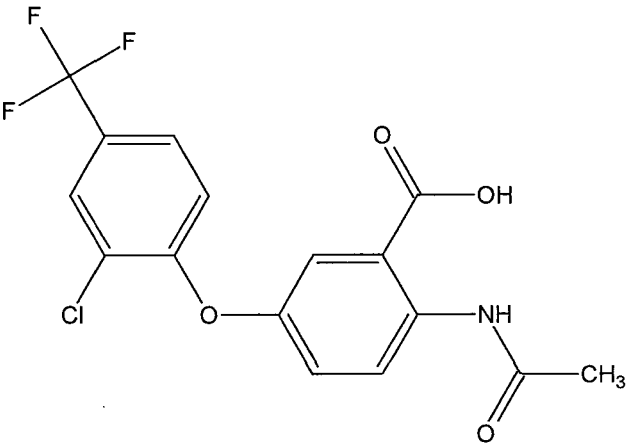
Compound Name : Acifluorfen (BAS9048)
CAS Number : 62476-59-9
IUPAC Name : 5-(2-chloro-4-trifluoromethylphenoxy)-2-nitrobenzoic acid
Molecular Formula : $C_{14}H_7ClF_3NO_5$
Molecular Weight : 361.7
Structure :



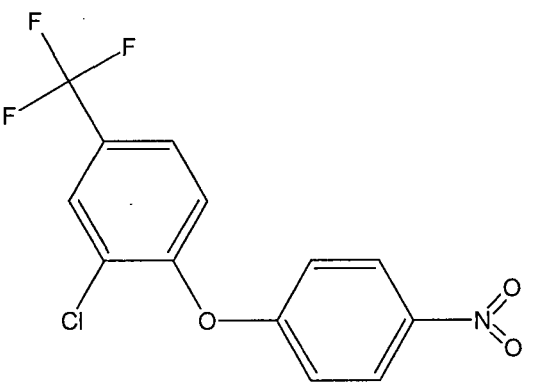
Compound Name : Acifluorfen Amine (BH 9048-A)
CAS Number : None
IUPAC Name : 5-(2-chloro-4-trifluoromethylphenoxy)-2-aminobenzoic acid
Molecular Formula : $C_{11}H_9ClF_3NO_3$
Molecular Weight : 331.7

Compound Name : Acifluorfen Acetamide (BH 9048-AA)
CAS Number : None
IUPAC Name : 5-(2-chloro-4-trifluoromethylphenoxy)-2-acetylaminobenzoic acid
Molecular Formula : $C_{16}H_{11}ClF_3NO_4$
Molecular Weight : 373.7

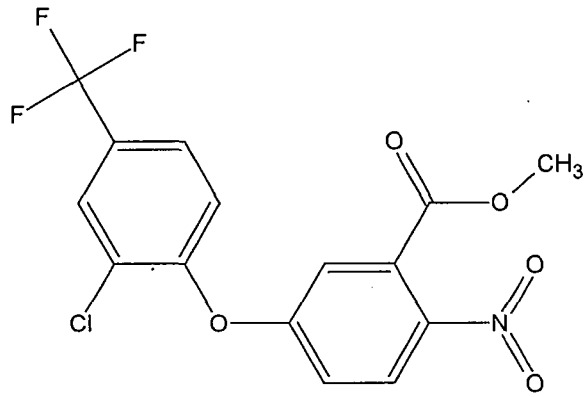
Structure :



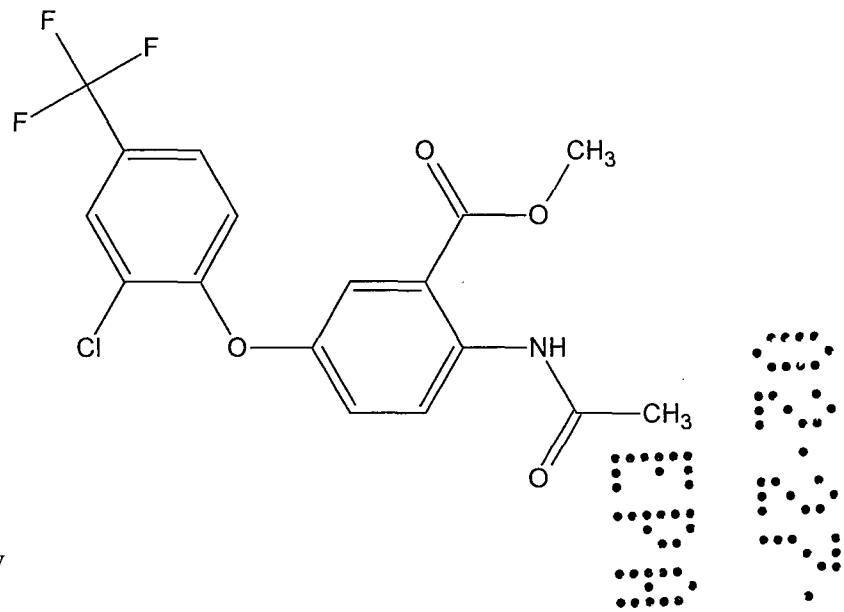
Compound Name : Des-carboxy Acifluorfen (BH 9048-DC)
CAS Number : None
IUPAC Name : 4-(2-chloro-4-trifluoromethylphenoxy)-nitrobenzene
Molecular Formula : $C_{13}H_7ClF_3NO_3$
Molecular Weight : 317.6
Structure :



Compound Name : Acifluorfen-Methyl-Ester (BH 9048-ME)
CAS Number : None
IUPAC Name : Methyl-5-(2-chloro-4-trifluoromethylphenoxy)-2-nitrobenzoate
Molecular Formula : $C_{15}H_9ClF_3NO_5$
Molecular Weight : 375.7
Structure :



Compound Name	:	Acifluorfen Acid Amide Methyl Ester (BH 9048-AAME)
CAS Number	:	None
IUPAC Name	:	Methyl-5-(2-chloro-4-trifluoromethylphenoxy)-2-acetylaminobenzoate
Molecular Formula	:	C17H13ClF3NO4
Molecular Weight	:	387.7
Structure	:	



1.2 Method Summary

BASF analytical method A9206 was developed for the analysis of Acifluorfen and its metabolites (Acifluorfen-Amine, Acifluorfen-Acetamide, and Des-Carboxy-Acifluorfen) in water. Water samples are homogenized by thoroughly shaking, and then acidified using 1N HCl. Acifluorfen and its metabolites are extracted by partitioning three times with dichloromethane/acetonitrile (1:1). The organic phase is passed through a phase separation paper to remove all traces of water, then concentrated and reconstituted in acetonitrile. An aliquot is removed for HPLC analysis for acifluorfen amine. The remaining sample is again concentrated and then methylated with trimethylsilyl diazomethane in hexane. The sample is reconstituted in toluene for GC analysis. Instrumental analysis is accomplished using an

HPLC with a fluorescence detector for acifluorfen-amine; and a GC/ECD for residues of des-carboxy-acifluorfen, methylated acifluorfen as acifluorfen-methyl-ester and acifluorfen-acetamide as acifluorfen acid amide methyl ester. The limit of quantification (LOQ) is 1 ppb for all analytes.

The BASF method A9206 was extended at JRF America in 2012 for the acifluorfen to an LOQ of 0.1 ppb (µg/L). The extraction procedure follows the original method, except that the derivatization step was eliminated. Analysis is accomplished on an HPLC/MS/MS system.

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g., glass distilled/HPLC grade solvents and analytical grade reagents. The water has to be deionized prior to use or purchased HPLC grade water. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Preparation of Analytical Standard Solutions

It is recommended that the following precautions should be taken when weighing the analytical materials.

1. Ensure good ventilation.
2. Wear gloves and laboratory coat.
3. Prevent inhalation and contact with
4. Wash any contaminated area

2.3.1 Stock Solutions

Stock standard solutions for acifluorfen, acifluorfen amine, acifluorfen acetamide and des-carboxy acifluorfen are prepared in acetonitrile; stock standard solution for acifluorfen methyl ester and acifluorfen acetamide methyl ester were prepared in toluene. All stock standards are given 6 months expiration date and stored in a refrigerator when not in use. The typical concentration for the stock standard is 100 µg/mL. The following is an example of the preparation of the acifluorfen stock standard.

1. Weigh ~ 0.0103 g of solid acifluorfen reference material (purity 99.1%) in a glass weigh boat and transfer to a 100 ml volumetric flask using acetonitrile.

2. Fill the volumetric flask halfway with acetonitrile and agitate gently (sonicate if necessary) until standards are completely dissolved.

3. Dilute to volume with acetonitrile and mix by inverting several times.

4. The concentrated stock solution will remain stable in a refrigerator at 0-7 °C for up to six months.

5. Calculate the exact concentration using the exact weight and purity as follows:

$$\frac{0.0103\text{g x } 99.1\% \text{ x } 1000000}{100\text{ mL}} = 102\ \mu g / mL$$

2.3.2 Preparation of Fortification Solutions

Fortification standard solutions containing acifluorfen, acifluorfen amine, acifluorfen acetamide and des-carboxy acifluorfen should be prepared by serial dilution of the stocks in acetonitrile. It is recommended that the following solutions are prepared: 0.1 µg/mL and 1.0 µg/mL in acetonitrile. Fortification standards are prepared fresh monthly when stored in a refrigerator.

2.3.3 Preparation of Calibration Standards for LC-MS/MS

Two sets of calibration solutions are prepared for the analysis. One set containing acifluorfen amine only is prepared in acetonitrile for HPLC analysis; another set containing acifluorfen methyl ester, acifluorfen acetamide methyl ester and des-carboxy acifluorfen is prepared in toluene for GC analysis. At least five levels of external calibration standards should be prepared to develop calibration curves for calculation of sample residues. The concentrations of calibration standards for HPLC analysis can be prepared as 2, 4, 10, 20 an 40 ng/mL in acetonitrile; and 20, 40, 100, 150, 200 and 400 ng/mL can be prepared for GC analysis. Typical dilution schemes used to prepare the GC calibration solutions are as follows:

GC Calibration Solutions

Starting Mixed Stock Concentration (µg/mL)	Volume Used (mL)	Final Volume (mL)	Final Concentration (ng/mL)
100	2.5 [1]	50	5.0 µg/mL
5.0	4.0	50	400
5.0	2.0	50	200
5.0	1.5	50	150
5.0	1.0	50	100
5.0	0.4	50	40
5.0	0.2	50	20

1- Volume of 2.5 mL of 100 µg/mL standard solution used for each stock standard of acifluorfen acetamide methyl ester and des-carboxy acifluorfen.

The calibration standards are stored refrigerated and given an expiration date of one month.

2.3.4 Standard Solution Storage and Expiration

All stock and standard solutions should be stored in a refrigerator or freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of six months for stock standard solutions is recommended and one month for all fortification and calibration standards.

2.4 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate MSDS or a monograph such as 'Hazards in the Chemical Laboratory', edited by S. G. Luxon, The Chemical Society, London.

All standards in their purest form should be considered a chemical hazard. All caution should be exercised when handling pure material or concentrated stock solutions. Avoid skin contact and inhalation. See Material Safety Data Sheet documentation accompanying standard shipment.

3.0 ANALYTICAL PROCEDURE

3.1 Sample Preparation

Samples should be removed from a refrigerator and allowed to reach room temperature before use. No other preparation is needed.

3.2 Sample Extraction and Clean-up

1. Transfer a 100-gram representative sample to a 250-mL separatory funnel. [A volume measurement may be used, as well.]

2. For the preparation of analytical recovery samples, fortify control samples in the extraction vessel by pipetting a known volume and concentration of the fortification standard onto the sample matrix. Swirl to mix the fortification standards into the sample(s).

3. Add 800 µL of 1N HCl to each funnel and swirl to mix the acid into the sample(s).

4. Add 40mL DCM/ACN (DCM/ACN 50:50) to sample funnel, shaking ~ 30 seconds thoroughly (vent several times) and then allow the mixture to settle 10 minutes to obtain two clear layers of solution.

5. Slowly drain the organic layer (lower layer), and pass it through a phase separation paper to a Rapidvap beaker.

6. Repeat step 4 and step 5 twice more, combine the organic layer in the beaker.

7. Evaporate the filtrate using a RapidVap at a temperature of 40°C until total volume less than 20 mL.

8. Transfer quantitatively sample to a 50 mL centrifuge tube with ACN and bring the total volume to 25 mL with ACN.

9. Remove 1.0 mL from sample tube to an HPLC vial for Amine analysis on HPLC.

10. Transfer sample back to the evaporation beaker and continue concentrating at 40°C until 0.5 to 1.0 mL left.

11. Derivatize sample with 10 mL of 0.02M TMS-diazomethane in hexane, add 2 mL of acetone to make sample homogeneous.

12. Allow sample to stand for 1 hour.

13. Add 800 µL of toluene and concentrate to just dryness on a RapidVap with a temperature of 40°C.

14. Reconstitute sample to 1 mL toluene.

15. Transfer sample to an HPLC vial or dilute sample with toluene for GC analysis.

3.3 Time Required for Analysis

The methodology is normally performed with a batch of 10 to 12 samples. One chemist can complete the analysis of one batch of samples (10-12) in a period of 16 working hours.

3.4 Method Stopping Points

The extraction procedure should be completed in a day. Acceptable method recoveries will validate any work flow interruptions. Samples may be stored in a refrigerator (~ 4° C) in sealed containers when the analysis cannot be completed in a single day.

3.5 Extended Method

The method was extended in 2012 by JRF America achieving a lower LOQ of 0.1 ppb for acifluorfen. The extended method uses the same original method, except the derivatization step (as listed in Section 3.2, step 11) was eliminated and a UPLC/MS/MS detection system was used for the analysis.

1. The deviatization step in the original method was not performed.
2. As acifluorfen was directly analyzed, the calibration standards were prepared as acifluorfen instead of acifluorfen methyl ester.
3. The analysis was performed using a UPLC/MS/MS system. The instrumental conditions are listed below:

Instrument: Waters Acquity UPLC with Applied BioSystem/MDS Sciex 4000 Q-trap® [UPLC/MS/MS system]
Applied Biosystems Analyst Software version 1.5.1

Waters Acquity UPLC Conditions

Column	Acquity BEH C18, 5.0 cm x 2.1 mm, 1.7 um
Column Oven Temperature	Ambient
Flow rate	0.3 mL min^{-1}
Injection volume	10 µL
Stop Time	5.00 minutes
Mobile phase	Solvent A = 0.1% formic acid in water Solvent B = 0.1% formic acid in methanol

Mobile Phase Program (linear gradient changes)

Time (min.)	% A	% B
0.00	70	30
0.20	70	30
2.00	10	90
3.00	10	90
3.1	70	30

Typical Analyte LC Retention Times:

Analyte	Approx. Retention Time (Minutes)
Aciflurofen	2.9

Acquisition Ions and Compound Dependent Parameters

Analyte	Mass Transition	Dwell	DP	CE	CXP
Acifluorfen	359.9→315.8	150	-55	-12	-15

Typical MS/MS Voltage Conditions Used

Ionization Mode:	Turbospray
Scan Type	MRM
Polarity	negative
Resolution Q1	unit
Resolution Q3	unit
Curtain gas (N_2)	30
GS1	20
GS2	50
CAD gas (N_2)	medium
Ion Spray (V)	-4200
Temperature (°C)	450
EP	-10

4.0 FINAL DETERMINATION

The instrument analysis is performed on a GC/ECD system. The following instrumentation and conditions can be used as a general guidance.

4.1 GC Instrument Description

GC/ECD: Hewlett-Packard 6890 Plus gas chromatograph equipped with an Agilent 7683 series injector and Hewlett-Packard 6890 Plus ECD detector. The system is controlled and the data processed by Agilent GC ChemStation™ Revision B.04.02.

GC Column J&W DB-5 capillary column, 1.0 micron film thickness, 30 meter x 0.32 mm internal diameter.

Column temperature program:	Initial value 130°C (~0.5 minutes); 1st program rate 30°C/ min to 250°C hold until ~6 min; 2nd program rate 30°C/ min to 290°C hold until ~1 min; 10°C/ min to 290°C hold until ~17.2 min; final run time ~30 minutes
Injection volume:	1 µL
Carrier Gas:	Helium – 2.8 mL/ min
Inlet Temperature:	275°C splitless
Column Head Pressure	16.5 psi
Detector Temperature:	350 °C

Analyte Typical Retention Time

Analyte	Approximate Retention Time (min.)
des-carboxy acifluorfen	7.5
Acifluorfen Methyl Ester	11.7
acifluorfen acetamide methyl ester	12.9

Note: The GC settings above should be used as guidelines only. For optimal results, a tune should be performed by the analyst.

4.2 HPLC Chromatography Conditions

Column:	Partishpere C18-ODS 3
Wavelength:	350 nm
Injection Volume:	20 µL
Mobile Phase:	A: methanol 100% at 1.0 mL/minute B: 2.56% acetic acid in water at 0.35 mL/minute

5.0 CALCULATION OF RESULTS

5.1 Multi Point Calibration Procedure

Acifluorfen and its metabolites may be calculated in µg/kg using multi point calibration procedure as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (for example, 50% LOQ to 10 x LOQ). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to each analyte. Calibration standard solutions should be interspersed throughout the analysis, after approximately five injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit (no weight, 1/x weighted and $1/x^2$ weighted are acceptable) and c is the intercept value.

Therefore:

$$x = \frac{y - c}{m}$$

e) Calculate the residues in the sample, expressed as µg/kg as follows:

$$\text{Residue (µg/kg)} = \frac{\text{Area} - \text{Intercept}}{\text{Slope}} \times \text{Dilution Factor x Conversion Factor}$$

$$\text{Dilution Factor} = \left(\frac{\text{Final Volume}}{\text{Sample Weight}} \right)$$

Conversion factor: 1.0 for amine
1.0 for des-carboxy acifluorfen
0.963 to convert to aciflurofen
0.964 to covert to aciflurofen acetamide

A. Example:

Analyte: Amine acifluorfen

A water sample was analyzed and the concentration in the final extract calculated from the calibration curve was 0.0248 ng/mL.

$$\text{D.F.} = \frac{25\,\text{mL}}{100\,\text{g}} = 0.25\,\text{mL/g}$$

$$\text{Results (ng/g)} = 12.4 ng/mL \times 0.25 mL/g = 3.10 ng/g$$

B. Recovery Results

The recovery factor, expressed as a percentage (Recovery %), is calculated using the following equation:

$$\text{Recovery}\,\% = \frac{\text{Residue Fortified (ppb)} - \text{Residue control (ppb)}}{\text{ppb analyte added}} \times 100$$

6.0 UNTREATED CONTROL AND RECOVERY SAMPLES

If untreated control samples are available, untreated control samples should be analyzed for each set of samples analyzed to verify that samples are free from analyte contamination. A minimum of one control should be analyzed with each batch of samples.

A total of two recovery samples (untreated samples accurately fortified with a known amount of acifluorfen and its three metabolites were suggested in each analytical set. The recovery levels should be run at the LOQ (0.5 µg/kg) and a higher level to encompass the treated sample results.

APPENDIX 1
APPARATUS

Recommended Suppliers

A. LC/MS/MS System

1. Waters Acquity UPLC system. Waters, Chromatograph Division of Millipore Corp., Milford, MA or equivalent.

2. Applied Biosystems API 4000 Qtrap mass spectrometer with Analyst™ software version 1.4.2.

B. Column: Supelco Ascentis Express C18, 2.1 x 75 mm, 2.7 µ

C. SPE vacuum manifold, Supelco, Bellefonte, PA

D. C18 SPE cartridge, 500 mg/6 cc, J.T. Baker.

E. Eppendorf adjustable pipettes, assorted sizes

F. Centrifuge, of a capacity capable of centrifuging 250ml centrifuge containers at 2000 to 3,500 rpm. IEC Model MP4R, International Equipment company or equivalent.

G. Balance, analytical, Mettler TA200 or equivalent.

H. Balance, top-loading, Mettler or equivalent.

I. Sonicator, Fisher Scientific.

J. Glassware

Graduated test tubes, 15 ml, 13 mm x 100 mm.
Nalgene bottle, 250 ml.
Graduated cylinders, 100 ml and assorted.
Beakers, various sizes.
Class A volumetric flasks, assorted sizes
Autosampler vials, Alltech Assoc., Inc., Deerfield, IL
Disposable test tube, 15 ml.
Glass filtration adaptor
Glass funnel
Pasteur pipettes

APPENDIX 2
REAGENTS

A. Solvents and Reagents.

1. HPLC grade solvents or better should be utilized. Other brands and grades of solvents may be substituted as long as they do not produce interferences with the chromatography.

   a. Acetonitrile, VWR, West Chester, PA
   b. Dichloromethane, VWR, West Chester, PA
   c. MilliQ water, VWR, West Chester, PA
   d. Methanol (MeOH), VWR, West Chester, PA

2. Reagents should be ACS grade or better. Other brands of ACS grade reagents may be substituted as long as they do not produce interferences with the chromatography.

   a. Hydrochloric acid, VWR, West Chester, PA
   b. Formic acid, VWR, West Chester, PA
   c. TMS-diazomethane 2.0 M solution, Sigmal-Aldrich, WI

3. Working Solutions

   a. 1N Hydrochloric acid: dilute 17.2 mL conc. HCl to 200 ml with water.

   b. Extraction solution (50/50 dichloromethane/acetonitrile): mix 500 ml of dichloromethane with 500 ml of acetonitrile.

   c. Mobile phase A: 0.1% formic acid in water: dilute 1 ml of formic acid to 1 liter with water.

   d. Mobile phase B: 0.1% formic acid in methanol: dilute 1 ml of formic acid to 1 liter with methanol.

   e. 30% acetonitrile/70% water: mix 30 ml of acetonitrile with 70 ml water.

   f. 30% methanol/70% water: mix 30 ml of methanol with 70 ml water.

   g. 0.02 M TMS-diazomethane solution: dilute 1 mL of 2.0 M TMS-diazomethane to 100 mL with hexane and mix.

APPENDIX 9

WATER METHOD FLOWCHART [NON-EXTENDED METHOD]

Transfer 100-gram sample to a 250-mL separatory funnel

↓

Add 800 µL of 1N HCl and 40 mL DCM/ACN 50:50

↓

Shake ~ 30 seconds and then allow the mixture to settle for 10 minutes

↓

Drain the organic layer into a Rapidvap beaker through a phase separation paper

↓

Repeat DCM/ACN partition twice more, combine the extracts

↓

Evaporate sample on a RapidVap at 40°C until less than 20 mL

↓

Transfer sample to a 50 mL centrifuge tube with ACN and bring the volume to 25 mL with ACN

↓

Remove 1.0 mL for Amine analysis on HPLC

↓

Transfer sample back to the evaporation beaker and continue concentrating at 40°C until 0.5 to 1.0 mL left.

↓

Derivatize sample by adding 10 mL of 0.02M TMS-diazomethane and 2 mL of acetone

↓

Allow sample to stand for 1 hour

↓

Add 800 µL of toluene and concentrate to just dryness on a RapidVap at 40°C.

↓

Reconstitute sample to 1 mL toluene

↓

Transfer sample to an HPLC vial or dilute sample with toluene for GC analysis

APPENDIX 10

WATER METHOD FLOWCHART
EXTENDED METHOD FOR ACIFLUORFEN

Transfer 100-gram sample to a 250-mL separatory funnel

↓

Add 800 µL of 1N HCl and 40 mL DCM/ACN 50:50

↓

Shake ~ 30 seconds and then allow the mixture to settle for 10 minutes

↓

Drain the organic layer into a Rapidvap beaker through a phase separation paper

↓

Repeat DCM/ACN partition twice more, combine the extracts

↓

Evaporate sample on a RapidVap at 40°C until less than 1 mL of extract remains

↓

Transfer sample to a 10 mL graduated test tube

↓

Rinse the beaker several times with ACN and add the rinsate to the test tube

↓

Dilute to final volume of 10 mL with ACN

↓

Dilute all samples and transfer to an HPLC vial

↓

Analyze using UPLC/MS/MS